

# The thin green line: sustainable bioenergy feedstocks or invaders in waiting

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Academic editor: J. Molofsky | Received 17 September 2014 | Accepted 10 January 2015 | Published 14 April 2015

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**Citation:** Smith LL, Allen DJ, Barney JN (2015) The thin green line: sustainable bioenergy feedstocks or invaders in waiting. NeoBiota 25: 47–71. doi: 10.3897/neobiota.25.8613

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## Abstract

Numerous fast growing and highly competitive exotic crops are being selected for production of renewable bioenergy. Tolerance of poor growing conditions with minimal inputs are ideal characteristics for bioenergy feedstocks, but have attracted concern for their potential to become invasive. *Miscanthus × giganteus* is one of the most promising bioenergy crops in the US, but grower adoption is hindered by high establishment costs due to sterility. Newly developed fertile tetraploid *M. × giganteus* may streamline cultivation while reducing establishment costs. However, fertile seed dramatically increases the potential propagule pressure, and thus probability of off-site plant establishment. To empirically evaluate the invasive potential of fertile *M. × giganteus* in the Southeastern US, we compared fitness and spread potential relative to ten grass species comprising 19 accessions under both high and low levels of competition and disturbance. We chose species known to be invasive in the US (positive controls: *Arundo donax*, naturalized *M. sinensis*, *M. sacchariflorus*, *Phalaris arundinacea*, *Sorghum halepense*) and non-invasive (negative controls; *Andropogon gerardii*, ornamental *M. sinensis*, *Panicum virgatum*, *Sorghum bicolor*, *Saccharum* spp.). This novel design allows us to make relative comparisons of risk among species with varying invasiveness. After three years of establishment and growth in Blacksburg, Virginia, neither aboveground disturbance nor interspecific weed competition influenced fitness for fertile *M. × giganteus* or our positive and negative control groups. Fertile *M. × giganteus* produced 346% and 283% greater aboveground biomass than our positive and negative species, respectively. However, fertile *M. × giganteus* produced 74% fewer inflorescences m<sup>-2</sup> than our positive controls and 7% and 51% fewer spikelets inflorescence<sup>-1</sup> than the positive and negative control species. After 18 months of growth, we observed the vegetative and seedling spread of three of our positive control species (*S. halepense*, *P. arundinacea*, and *M. sacchariflorus*) outside the cultivated plot



into receiving areas of both high and low competition. After 24 months of growth, numerous species were observed outside the cultivated plot including fertile *M. × giganteus* and 50% of negative control species. Notably, in three years sterile *M. × giganteus* ‘Illinois’ and *Arundo donax* never moved from the cultivated plot. The addition of fertile seed appears to increase the potential for offsite movement, but within the geographic confines of our empirical evaluation, fertile *M. × giganteus* seedlings are more similar to native *P. virgatum* and were not nearly as fast growing or as competitive as our positive control *S. halepense*. The use of numerous species providing relative comparisons allow us to draw important conclusions which may help prepare for widespread commercialization, while providing novel methodology for ecological risk assessment of new species.

### Keywords

Biofuel, giant miscanthus, habitat susceptibility, invasibility

### Introduction

There is a global push towards renewable biomass based energy (Yauan et al. 2008), and large statured perennial grasses hold the most promise as dedicated energy crops. Candidate feedstocks are ideal because of their perennial growth habit, rapid growth and high annual biomass production, low management and input requirements following establishment, and relatively low pest pressure. However, it is this desirable set of agronomic characteristics which has been the major source of concern for their potential to contribute to the invasiveness of numerous bioenergy crops (Lewandowski et al. 2003; Raghu et al. 2006). Barney and DiTomaso (2010) diagram the “thin green line” between many agronomic weeds, introduced and even subsidized in some cases, for purposes such as forage or erosion control, and the relatively benign crops vitally important to our economy and food supply. However, identifying which side of this line new crops fall is challenging at best.

Spatial demographic models (Matlaga and Davis 2013) and weed risk assessments (Barney and DiTomaso 2008; Davis et al. 2010; Gordon et al. 2011) do offer predictions regarding the ability of novel species to establish and spread. However, in reality, empirical data from in situ field trials, supporting conclusions of invasiveness or long-term sustainability, do not exist. Several candidate bioenergy crops have a history of invasiveness, which is a robust predictor of future invasive potential (Dawson et al. 2009; Gordon et al. 2008). For example, *Arundo donax* L. is a documented noxious weed of riparian habitats in the southeastern United States (Bell 1997; Katibah 1984). Despite this label, no peer-reviewed data exists evaluating the ability of *A. donax* to spread from a cultivated field. Two miscanthus species in particular have been widely grown in the United States for horticultural use: *Miscanthus sinensis* Andersson is listed as potentially invasive, but not prohibited in Connecticut (Council 2013), and is known to form extensive infestations after spreading from older or abandoned ornamental plantings (Dougherty et al. 2014; Miller 2003); and *M. sacchariflorus* (Maxim.) Franch is on the Massachusetts prohibited plant list (Resources 2014), and has repeatedly escaped from cultivation, particularly in the Midwest (Bonin et al. 2014). However, at this time only



limited studies have begun to examine the invasive potential of bioenergy crops in the context of a managed agricultural cropping system (see Barney et al. 2012; Matlaga et al. 2012b; Quinn et al. 2011).

Crop breeding and improvement will be imperative to improve quality, increase yield, and reduce pest pressure (Gressel 2008). The sterile triploid 'Illinois' variety of *Miscanthus*  $\times$  *giganteus* J.M. Greef & Deuter ex Hodkinson & Renvoize has emerged as one of the most promising bioenergy crops in the US and Europe, but planting is expensive and requires specialized equipment (Lewandowski et al. 2003). Despite the explicit inclusion of *M.*  $\times$  *giganteus* in the Massachusetts prohibited plant list as a progeny of *M. sacchariflorus* (Resources 2014), qualitative weed risk assessments have suggested that the sterile cultivar is of low risk for invasiveness (Barney and DiTomaso 2008). Newly developed fertile lines of tetraploid *M.*  $\times$  *giganteus* may streamline cultivation by reducing labor and establishment costs (Sacks et al. 2013); but the addition of fertile seed has the potential to dramatically increase propagule pressure to surrounding habitats, and must be evaluated for its influence on invasiveness.

Despite the vigilant approach with bioenergy crops in regards to invasiveness, the majority of introduced species have neutral ecological consequences and many provide a direct benefit to society (Barney and DiTomaso 2010; Davis 2003). However, the intentional cultivation and transport of exotic bioenergy crops over a vast geographic range would bypass the early environmental filters of introduction and colonization (Barney et al. 2012), as well as the geographical, environmental and reproductive barriers to spread (Richardson and Blanchard 2011; Smith and Barney 2014b). Both vegetative and seed propagules from bioenergy crops will be exposed to a diversity of landscapes along the biofuel supply chain (cultivated field to refinery). Therefore, susceptibility to invasion will need to be evaluated across numerous geographies and habitat types (Smith and Barney 2014b).

Since Herbert Baker (1965; 1974) put forth the theory that a set of 12 defining characteristics could identify the 'ideal weed', the importance of traits has been widely debated (Thompson and Davis 2011; van Kleunen et al. 2010). Bioenergy crops have been selected for a suite of agronomic traits making them ideal candidates for cultivation (Lewandowski et al. 2003), but this may also serve as the crux for their potential to become invasive (Raghu et al. 2006). For these reasons, it is imperative that we reflect that no single species is invasive in every location it inhabits. For example, populations of *Sorghum halepense* L. are particularly devastating in the southeastern United States, earning a reputation as one of the world's worst weeds (Holm et al. 1977). Yet in its northern range, *S. halepense* populations are rarely regarded as detrimental and despite its perennial growth many populations do not overwinter as rhizomes (Warwick et al. 1984), illustrating that both invasiveness and habitat susceptibility vary (Smith and Barney 2014b).

Here we use a comparative framework to relativize the invasive potential of newly developed fertile tetraploid *M.*  $\times$  *giganteus*. We compare fertile *M.*  $\times$  *giganteus* against ten grass species, comprising 19 accessions, in four environments. We selected the ten grass species to allow a comparison against species that are known invaders in the



US (positive controls), and species that are generally considered not to be invasive (negative controls). This design allows us to make important relative comparisons of risk, for candidate bioenergy crops, along a spectrum of invasiveness. We impose both competition and aboveground disturbance treatments to capture a range of conditions which bioenergy crops may encounter in or adjacent to the cultivated field. These treatments allow us to determine conditions that facilitate invasive spread and determine susceptible environments for establishment of nascent populations. This relative methodology was recently tested and proved critical in accurately interpreting the probability of fertile tetraploid *M. × giganteus* establishment in a diversity of habitats across the southeastern United States (Smith and Barney 2014b). The objective of this study is to compare the growth and spread potential of fertile *M. × giganteus* to known invasive and noninvasive control groupings. Specifically, we aim to: (1) evaluate the invasive potential of fertile *M. × giganteus*, during the first three years of establishment and growth, in comparison with 10 species (19 total accessions) of known invasive and non-invasive species in relation to their population dynamics, competitive ability, local recruitment and spread potential; (2) evaluate the performance of each bioenergy crop in response to various levels of competition and disturbance by assessing survival and performance; and (3) quantify seed production as a novel propagule source for *M. × giganteus* and compare across our invasiveness diversity panel.

## Methods

### Species selection

In our effort to evaluate the invasive potential of a new fertile tetraploid *M. × giganteus* pre-commercial cultivar known as ‘PowerCane’ ((Sacks et al. 2013); Mendel Biotechnology, Inc., Hayward, CA, USA), which was derived from a population of half-siblings of ‘Nagara’ (Głowacka et al. 2014), we developed a methodology that includes several species that are known invaders in the southeastern US (hereafter positive controls), as well as species that are generally considered not invasive (hereafter negative controls). In order to make relative comparisons of fertile *M. × giganteus*, we selected a range of grasses to represent various growth habits (clumping to spreading) and fertilities that span this invasive spectrum, and that occur regionally (Table 1). Weedy populations of all positive controls are well documented regional invaders (Table 1), and many receive a high risk rating from the Plant Protection and Quarantine Weed Risk Assessment Model (Koop et al. 2011; Smith 2014). Negative controls have been documented as low risk in weed risk assessment models (PIER 2013; Smith 2014), or are native to North America and are not considered highly competitive or weedy in comparison to our positive controls (Simberloff et al. 2012). In some cases multiple accessions (e.g., *M. sinensis*) or cultivars (e.g., *P. virgatum*) are included to represent intraspecific variation (Table 1). Despite the weedy reputation of *M. sinensis* (Dougherty et al. 2014; Miller 2003) we selected accessions intended to represent both positive and negative



**Table 1.** List of taxa included in the field trials located in Blacksburg, Virginia.

Species	Common name	Accession	Source	Planting method	Planting format <sup>§</sup>	Invasive status in the US
<i>Andropogon gerardii</i>	big bluestem	Suther	Ernst	seed	16.5 R	native <sup>l</sup>
<i>Arundo donax</i>	giant reed		Bluemel	plugs	76 C	invasive <sup>†</sup>
<i>Miscanthus sacchariflorus</i>	Amur silvergrass	Robustus	Bluemel	plugs	76 C	invasive <sup>#</sup>
<i>M. sinensis</i>	maiden grass	Gracillimus	Bluemel	plugs	76 C	exotic <sup>††</sup>
<i>M. sinensis</i>	maiden grass	Dixieland	Bluemel	plugs	76 C	exotic <sup>††</sup>
<i>M. sinensis</i>	maiden grass	Cabaret	Bluemel	plugs	76 C	exotic <sup>††</sup>
<i>M. sinensis</i>	maiden grass	OH	UIUC	plugs	76 C	invasive <sup>‡‡</sup>
<i>M. sinensis</i>	maiden grass	NC	UIUC	plugs	76 C	invasive <sup>‡‡</sup>
<i>M. sinensis</i>	maiden grass	KY	UIUC	plugs	76 C	invasive <sup>‡‡</sup>
<i>M. sinensis</i>	maiden grass	PA	UIUC	plugs	76 C	invasive <sup>‡‡</sup>
<i>M. × giganteus</i>	giant miscanthus	Illinois	Mendel	plugs	76 C	exotic <sup>§§</sup>
<i>M. × giganteus</i> <sup>†</sup>	PowerCane	M700464	Mendel	plugs	76 C	unknown
<i>M. × giganteus</i>	giant miscanthus	Nagara	Mendel	plugs	76 C	exotic <sup>§§</sup>
<i>Panicum virgatum</i>	switchgrass	EG 1101	Ceres	seed	16.5 R	native <sup>l</sup>
<i>P. virgatum</i>	switchgrass	Alamo	Ernst	seed	16.5 R	native <sup>l</sup>
<i>Phalaris arundinacea</i>	reed canarygrass	Palaton	Outsidepride	seed	16.5 R	invasive <sup>  </sup>
<i>Saccharum</i> spp. <sup>‡</sup>	energy cane	US 06-9001	USDA-ARS	ratoons	76 C	exotic <sup>¶¶</sup>
<i>Saccharum</i> spp.	energy cane	US 06-9002	USDA-ARS	ratoons	76 C	exotic <sup>¶¶</sup>
<i>Sorghum bicolor</i>	biomass sorghum	ES 5201	Ceres	seed	76 R	exotic <sup>##</sup>
<i>S. halepense</i>	johnsongrass		Azlin	seed	16.5 R	invasive <sup>†††</sup>

<sup>†</sup> Following the nothospecies rule from the International Code of Nomenclature for algae, fungi, and plants (IAPT 2012), all progeny and other descendants derived from crossing *M. sacchariflorus* and *M. sinensis* are by definition *M. × giganteus*, irrespective of ploidy and fertility.

<sup>‡</sup> Due to issues with availability, *Saccharum* cultivars were planted in summer 2012, one year later than all other species.

<sup>§</sup> Planting format in 16.5 cm (16.5 R) or 76 cm (76 R) rows, or on 76 cm centers (76 C).

<sup>l</sup>(Simberloff et al. 2012); <sup>†</sup>(Bell 1997); <sup>#</sup>(Bonin et al. 2014); <sup>††</sup>(Madeja et al. 2012); <sup>‡‡</sup>(Dougherty et al. 2014); <sup>§§</sup> (Heaton et al. 2004); <sup>||</sup>(Kercher et al. 2007); <sup>¶¶</sup> (Gordon et al. 2011); <sup>##</sup> (Martin et al. 2006);

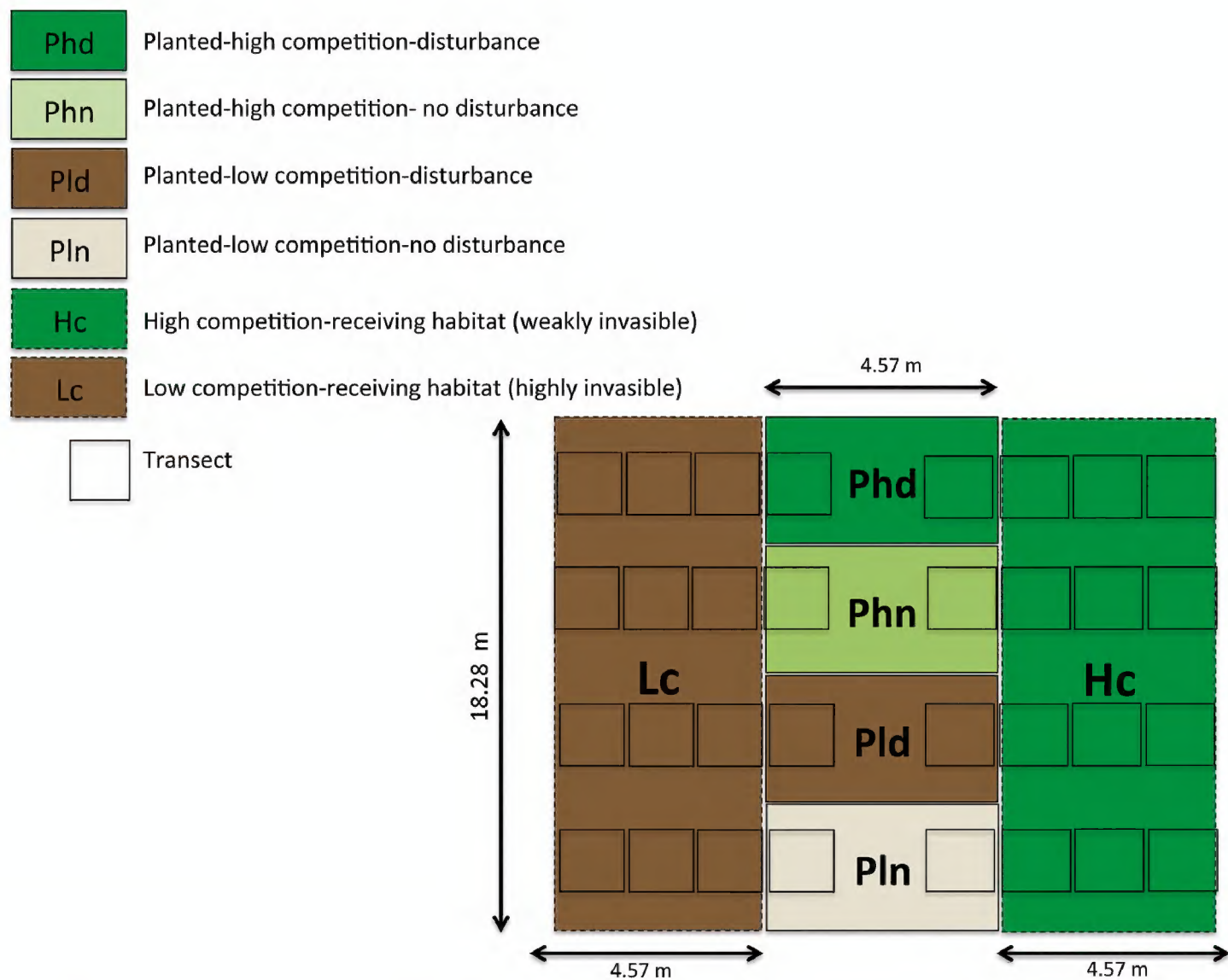
<sup>†††</sup> (Holm et al. 1977).

controls. Several *M. sinensis* ornamental cultivars are poor horticultural performers with low fecundity (Madeja et al. 2012), hence our characterization of low invasive potential in this study.

## Experimental design

A two-factor split-plot design arranged in a randomized complete block, with four replications was established in Blacksburg, Virginia, Schochoh, Kentucky and Auburn, Alabama in 2011. A total of 20 13.7 × 18.3 m plots were established for each accession with the exception of the four naturalized *M. sinensis* accessions, which were replicated three times at each site due to seed limitation. Within each plot we planted the center





**Figure 1.** A single replicate, showing the two-factor split-plot layout for each of the 76 plots established in Blacksburg, VA.

4.6 × 18.3 m with the target taxon, which is flanked by an equally-sized receiving area on either side. The planted plot was divided into four 4.6 × 4.6 m subplots (20.88 m<sup>2</sup>) randomly assigned to one of the following treatments (Figure 1): high competition/ no disturbance (Phn); high competition/ disturbance (Phd); low competition/ no disturbance (Pln); low competition/ disturbance (Pld). The disturbance treatment refers to annual aboveground biomass removal in the fall of each year (beginning December 2011), while a no disturbance treatment is defined by the absence of annual harvest, in which all plant material was left standing in the field for the duration of this study. The high competition treatment refers to no weed management action taken following initial establishment (after July 2011). High competition plots included dense populations of both winter (i.e., *Lamium* sp. and *Stellaria* sp.) and summer annual weeds (i.e., *Ipomoea* sp., *Amaranthus* sp., *Setaria* sp., and *Conyza canadensis* (L.) Cronquist). Low competition is defined as intensive weed management to maintain a near weed-free (~bare ground) environment throughout the duration of the experiment. Each flanking unplanted 4.6 × 18.3 m “receiving habitat” was also randomly assigned to either intensive management for weeds (Lc) or no management (Hc) (Figure 1), to assess the local spread of each species as a function of habitat type. The Hc receiving areas



were composed of the same winter and summer annual weeds as above. Weedy plots represent a relatively high competition habitat, while the weed-free plots represent a relatively low competition habitat. Each plot was surrounded with a 1.5 m buffer of mowed *Schedonorus arundinaceus* (Schreb.) Dumort (tall fescue).

## Site

This experiment was established at the Kentland Research Farm near Blacksburg, VA USA (37°12'N, 80°35'W), on 10 June 2011, Walnut Grove Farms, Schochoh, KY (36°45'N, 86°45'W) on 15 June 2011, and Auburn, Alabama on 30 May 2011 (32°26'N, 85°52'W). However, due to unforeseen circumstances beyond our control, the Kentucky and Alabama locations were eradicated within the first year of the study. Therefore, neither will be discussed further. We understand the limitations of a single geographic location in years two and three of this study; but due to the proprietary nature of the 'PowerCane' and 'Nagara' plant material, we were limited by site availability. It should be noted that other important ecological studies have been carried out using a single location (Von Holle 2005; Von Holle and Simberloff 2005). However, our results should be viewed within the limited geographic representation. Soil samples were collected in early June 2011, using a 20 cm<sup>3</sup> soil corer, and submitted for analysis at the Virginia Cooperative Extension Soil Testing Laboratory at Virginia Tech. The Blacksburg field site was planted on a Ross loam occluding a Wheeling silt loam (USDA-NRCS 2013) with a pH of 6.4, and a recent cropping history of continuous corn with a winter rye cover crop. In May 2011 the field was treated with 1 kg acid equivalent ha<sup>-1</sup> glyphosate. The rye cover crop was mowed and bailed in preparation for planting.

## Establishment

Vegetatively propagated accessions (Table 1) were started in the greenhouse from rhizome/root crown fragments and delivered to the field sites just prior to planting. Seed from naturalized accessions of *M. sinensis* were collected the previous year from established populations in Kentucky, Pennsylvania, North Carolina and Ohio. Seeds were planted individually into 127 cell flats in March 2011 and greenhouse grown. The field was not tilled prior to planting in accordance with a no-till cropping system. Miscanthus plugs were planted on 76 cm centers using a no-till plug planter (RJ Equipment, Ontario, Canada). Larger plant material such as *A. donax* required hand planting, because pieces were too large for the transplanting equipment. All remaining seeded feedstocks were drilled on 16.5 or 76 cm rows using a no-till drill (Table 1). Seeding rate was adjusted based on commercially determined pure live seed or (laboratory) germination rates, to deliver ~22,000 plants ha<sup>-1</sup>, which was comparable to our transplant density. As our study aims to evaluate establishment, persistence and spread,



annual *Sorghum bicolor* L. was not replanted annually after June 2011. *Saccharum* US 06-9001 and US 06-9002 were not planted until May 2012, due to delays in germ-plasm availability.

To improve stand establishment, the entire field site received 350 g ai ha<sup>-1</sup> and 822 g ai ha<sup>-1</sup> 2,4-D on July 6 and July 25 respectively. Following the July herbicide application we decided that sufficient time for seedling/plug establishment had elapsed, and thus no further herbicide applications were made in the high competition plots (Phn and Phd). No herbicide treatments were imposed in the high competition receiving habitat (Hc). Herbicide treatments of 1060 g ai ha<sup>-1</sup> 2,4-D plus 560 g ai ha<sup>-1</sup> dicamba were sprayed on August 20, 2011, in the low competition plots (Pld and Pln) and low competition receiving habitats (Lc). Supplemental hand weeding was done as needed. A second treatment of 2,4-D and dicamba (1060 g ai ha<sup>-1</sup> and 560 g ai ha<sup>-1</sup> respectively) was applied on September 25. Low competition plots received 1680 g ai ha<sup>-1</sup> atrazine at the beginning of the second and third growing seasons. Herbicide treatments of 1060 g ai ha<sup>-1</sup> 2,4-D plus 560 g ai ha<sup>-1</sup> dicamba and 31.5 g ai ha<sup>-1</sup> halosulfuron including a 1% v/v nonionic surfactant were applied approximately once a month to maintain weed free status within plots and in the low competition receiving habitat. A 1 kg ae ha<sup>-1</sup> application of glyphosate was also used to selectively spot treat non-target grass weeds when hand weeding was not time effective.

## Measurements

Spring data collection occurred in May of 2012 and was repeated in May 2013, while fall data collection occurred in November of each year prior to harvest. To characterize population demography, seedling recruitment and individual plant performance, we placed two 0.9 × 1.2 m quadrats in the middle of each sub-plot adjacent to the receiving habitat (Figure 1). Previous germination studies of *M. × giganteus* ‘Illinois’ failed to yield evidence of fertile seed (Matlaga et al. 2012b); thus, any *Miscanthus* seedlings observed within our plots of *M. × giganteus* ‘Illinois’ and ‘Nagara’, both of which are sterile, were broadly designated *Miscanthus* spp. and assumed to have moved beyond the 4.6 × 18.3 m receiving area of *Miscanthus* spp. Due to the large number of visually identical *Miscanthus* spp. seedlings and lack of available tools for determining genetic lineage, we made the assumption that any *Miscanthus* seedling found within a seed bearing *Miscanthus* (*M. sinensis* or ‘PowerCane’) plot or receiving area was the progeny of plants associated with that specific plot. The same assumption was also made for the two cultivars of *P. virgatum*. Measurements include plant density, canopy height to the tallest node, culm number per plant, number of inflorescences per plant and basal plant diameter. After two growing seasons the habit of *S. halepense*, *M. sacchariflorus* and *P. arundinacea* made it impossible to distinguish vegetative ramets from adjacent plants or seedlings, hence our need to base all measurements on culms per unit area rather than the number of individuals. In the receiving habitats, data was collected in



three  $0.9 \times 1.2$ -m quadrats arranged as a transect perpendicular to the planted plot, adjacent to the planted area until the end of the 4.57 m receiving habitat (Figure 1). The same metrics as above were collected for newly emerged plants found in the receiving habitat (of any of the 20 accessions in this study). Five inflorescences were randomly harvested from each subplot in November 2013. Total spikelet number for each inflorescence was recorded with the exception of the sterile *A. donax*, *M. × giganteus* ‘Illinois’, and *M. × giganteus* ‘Nagara’ (Mariani et al. 2010; Matlaga et al. 2012a). Three replications of 100 seeds for each “fertile” accession were surface sterilized with 10% bleach for 30 seconds and rinsed with deionized water. Seeds were then placed on germination paper in  $9.5 \times 9.5$  cm sealed petri plates with ~10 ml deionized water and germination was monitored for four weeks. Harvest treatments were imposed from November to January in each year as weather permitted.

## Follow up

Upon termination of the experiment, the entire field was sprayed with 2 kg ae ha<sup>-1</sup> glyphosate in late 2013 and early 2014. All plant material was harvested, removed from the site and burned as was done with the harvested material in 2012 and 2013. In late summer 2014 we applied 7 L ha<sup>-1</sup> imazapyr. Glyphosate-tolerant corn or soybeans will be planted in the spring of 2015, and a three-year scouting and weed management plan will be implemented to ensure all propagules have been removed from the site. It should be noted that no individuals of any species have been detected outside the experimental area to date.

## Statistical analysis

Analysis of variance (ANOVA) was performed on fitness parameters using JMP 10 statistical software (SAS Institute, Cary, North Carolina, USA). Aboveground biomass, height, culm number, inflorescence number, seed number, and seedling density were analyzed as a mixed model. Treatments and accessions are considered fixed effects, with the 20 accessions nested within designated invasiveness groups (positive and negative controls), while blocks were considered a random effect. Numerous transformations were performed, depending on measurement and year, to achieve normality of residuals. All interactions varied by year, and we were only interested in within year comparisons. Therefore, we did not perform a repeated measures analysis, and look at the variance structure within each of the three years. When significant treatment effects occurred, means were compared with Tukey-Kramer test at  $\alpha < 0.05$ , or when more complex interactions were significant, means were compared with a priori orthogonal contrasts at  $\alpha < 0.05$ . The 20 individual accessions in our study had an underlying structure (invasiveness groupings), central to our



experimental design. To objectively determine if our measured traits were capable of partitioning the accessions into the invasiveness groups we performed a canonical discriminant analysis. Kenkel et al. (2002) suggest canonical discriminant analysis is appropriate to examine the relationships between our transformed fitness metrics height, culm and inflorescence number, and spikelet production (covariates) and our invasiveness response variables.

## Results

### Establishment (year 1)

Growth in the first year of all 19 perennial grasses was low as expected. Establishment was well below our target density of 22,000 plants ha<sup>-1</sup> for the negative controls *A. gerardii* and ornamental cultivars of *M. sinensis*. Despite heavy and uniform weed pressure in weedy plots, competition had no influence on aboveground biomass, culms m<sup>-2</sup>, height or inflorescences m<sup>-2</sup> (Table 2). *Miscanthus* × *giganteus* ‘PowerCane’ (‘Powercane’ hereafter) and our positive control group were taller ( $P < 0.001$ ) and produced more culms m<sup>-2</sup> ( $P < 0.001$ ) than the negative control group (Figure 2). The negative control group did produce 46% and 84% greater aboveground biomass than ‘PowerCane’ and the positive control group respectively. Alternatively, our positive control group produced eight-fold more inflorescence m<sup>-2</sup> than ‘PowerCane’ and our negative controls, which did not differ from one another. *Sorghum bicolor* (negative control), the only annual species in our trial, was taller ( $244 \pm 15$  cm) and had significantly greater aboveground biomass ( $15 \pm 6$  Mg ha<sup>-1</sup>) than all other taxa ( $P = 0.0014$ ). *Sorghum halepense* (positive control) produced the greatest number of inflorescences ( $74.9 \pm 6.4$  m<sup>-2</sup>) in the first year, while ‘PowerCane’ produced only  $1.3 \pm 0.2$  inflorescences m<sup>-2</sup>. None of the 20 accessions evaluated here spread outside the cultivated plot into either adjacent receiving area after one growing season.

### Year 2

By 12 months after planting, two culms of *M. sacchariflorus* were observed to have spread into the high competition (Hc) receiving area; no culms were found in the low competition (Lc) receiving area. *Sorghum halepense* spread extensively 0 and 1.5 m into both the Lc ( $198 \pm 18$  culms m<sup>-2</sup>) and Hc ( $152 \pm 33$  culms m<sup>-2</sup>) receiving areas.

In fall 2012, *M. × giganteus* ‘PowerCane’ was taller ( $267.5 \pm 11.6$  cm) and produced more culms ( $130 \pm 22$  m<sup>-2</sup>) than negative controls and had greater aboveground biomass ( $26 \pm 3$  Mg ha<sup>-1</sup>) than the positive and negative control groups (Figure 2). Competition treatments influenced biomass, culm number, and height, while disturbance had no effect on any measured parameter (Table 2). However, competition from unmanaged weeds did not reduce ‘PowerCane’ height ( $P = 0.1571$ ), culm number



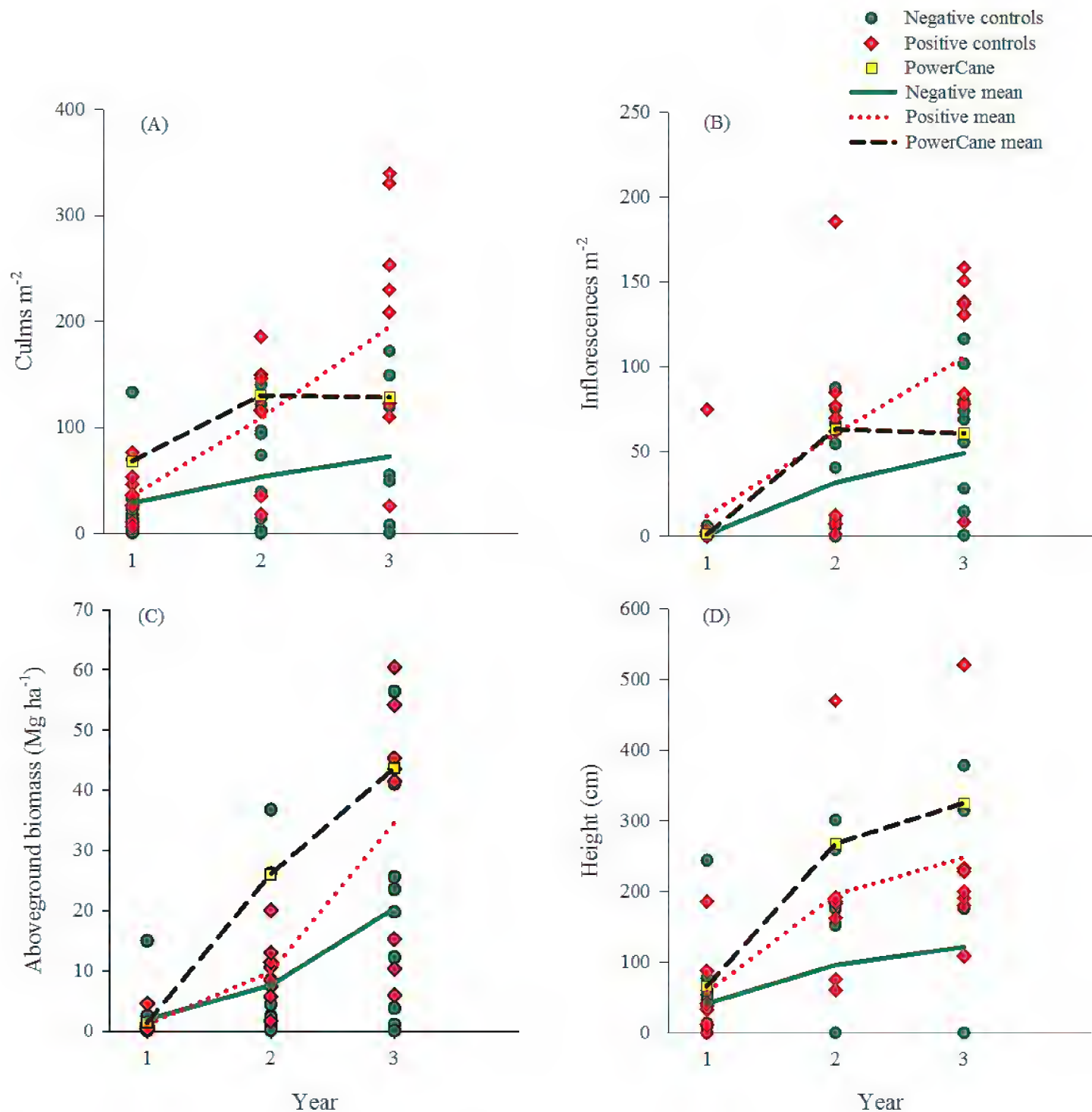
**Table 2.** Results of a mixed model ANOVA to evaluate competition and disturbance on aboveground biomass, culm number, number of inflorescences, and height for 20 accessions nested within invasiveness groups observed over three growing seasons in Blacksburg, VA.

		df	Biomass	df	Culm number	Height	Inflorescence number
Year 1	Block	3	0.2267	3	0.1104	0.3410	<b>0.0093</b>
	Species invasiveness	2	<b>&lt;.0001</b>	2	<b>&lt;.0001</b>	<b>&lt;.0001</b>	<b>&lt;.0001</b>
	Species (species invasiveness)	15	<b>&lt;.0001</b>	19	<b>&lt;.0001</b>	<b>&lt;.0001</b>	<b>&lt;.0001</b>
	Competition	1	0.7651	1	0.1297	0.9889	0.5704
	Competition × species invasiveness	2	0.2267	2	0.1482	0.8867	0.9010
Year 2	Block	3	0.3444	3	0.1365	0.4277	0.2615
	Species invasiveness	2	<b>&lt;.0001</b>	2	<b>&lt;.0001</b>	<b>&lt;.0001</b>	<b>&lt;.0001</b>
	Species (species invasiveness)	17	<b>&lt;.0001</b>	17	<b>&lt;.0001</b>	<b>&lt;.0001</b>	<b>&lt;.0001</b>
	Competition	1	<b>0.0475</b>	1	<b>0.0087</b>	0.8145	<b>0.0003</b>
	Disturbance	---	---	1	0.8393	0.5382	0.9390
	Competition × species invasiveness	2	<b>0.0205</b>	1	0.9876	<b>0.0411</b>	0.8763
	Disturbance × species invasiveness	---	---	2	0.9258	0.3361	0.1236
	Competition × disturbance	---	---	1	0.5873	0.8950	0.9295
	Competition × disturbance × species invasiveness	---	---	2	0.9879	0.5629	0.2624
Year 3	Block	3	0.6655	3	0.3943	<b>0.0021</b>	0.5631
	Species invasiveness	2	<b>&lt;.0001</b>	2	<b>&lt;.0001</b>	<b>&lt;.0001</b>	0.0818
	Species (species invasiveness)	16	<b>&lt;.0001</b>	17	<b>&lt;.0001</b>	<b>&lt;.0001</b>	<b>&lt;.0001</b>
	Competition	1	0.2491	1	0.2686	0.3401	0.5298
	Disturbance	1	0.1074	1	0.8797	0.2327	0.1519
	Competition × species invasiveness	2	0.1041	1	0.4864	0.5878	0.5585
	Disturbance × species invasiveness	2	0.0742	2	0.6574	0.7645	0.6781
	Competition × disturbance	1	0.3392	1	0.5440	0.4407	0.8374
	Competition × disturbance × species invasiveness	2	0.8225	2	0.6979	0.4895	0.8232

( $P=0.3867$ ) or biomass ( $P=0.2928$ ). Competition reduced biomass 56% and 70% and culm numbers 26% and 53% of positive and negative control groups, respectively. Interestingly, inflorescence production was not influenced by competition, with positive controls and ‘PowerCane’, both producing a mean of 62 inflorescences  $m^{-2}$ , 95% more than negative controls (Figure 2). *Sorghum halepense* again produced the greatest number of inflorescences ( $186 \pm 15 m^{-2}$ ) among the positive control group, 118% more than naturalized *M. sinensis* PA, which ranked second for inflorescence production.

No further spread of *M. sacchariflorus* was observed between the spring and fall 2012. *Phalaris arundinacea* was observed in the Lc receiving area with  $2.7 \pm 2.1$  culms  $m^{-2}$  at a distance of 0 to 1.5 meters from the planted plot. Population density of *S. halepense* continued to increase from year one to year two in the Lc and Hc receiving areas. At the 3 to 4.5 m distance, culm number increased by 584% (from 19 to  $130 \pm 17$  culm  $m^{-2}$ ) in the Lc receiving area and 420% (from 5 to  $26 \pm 7$  culms  $m^{-2}$ ) in the Hc receiving area.





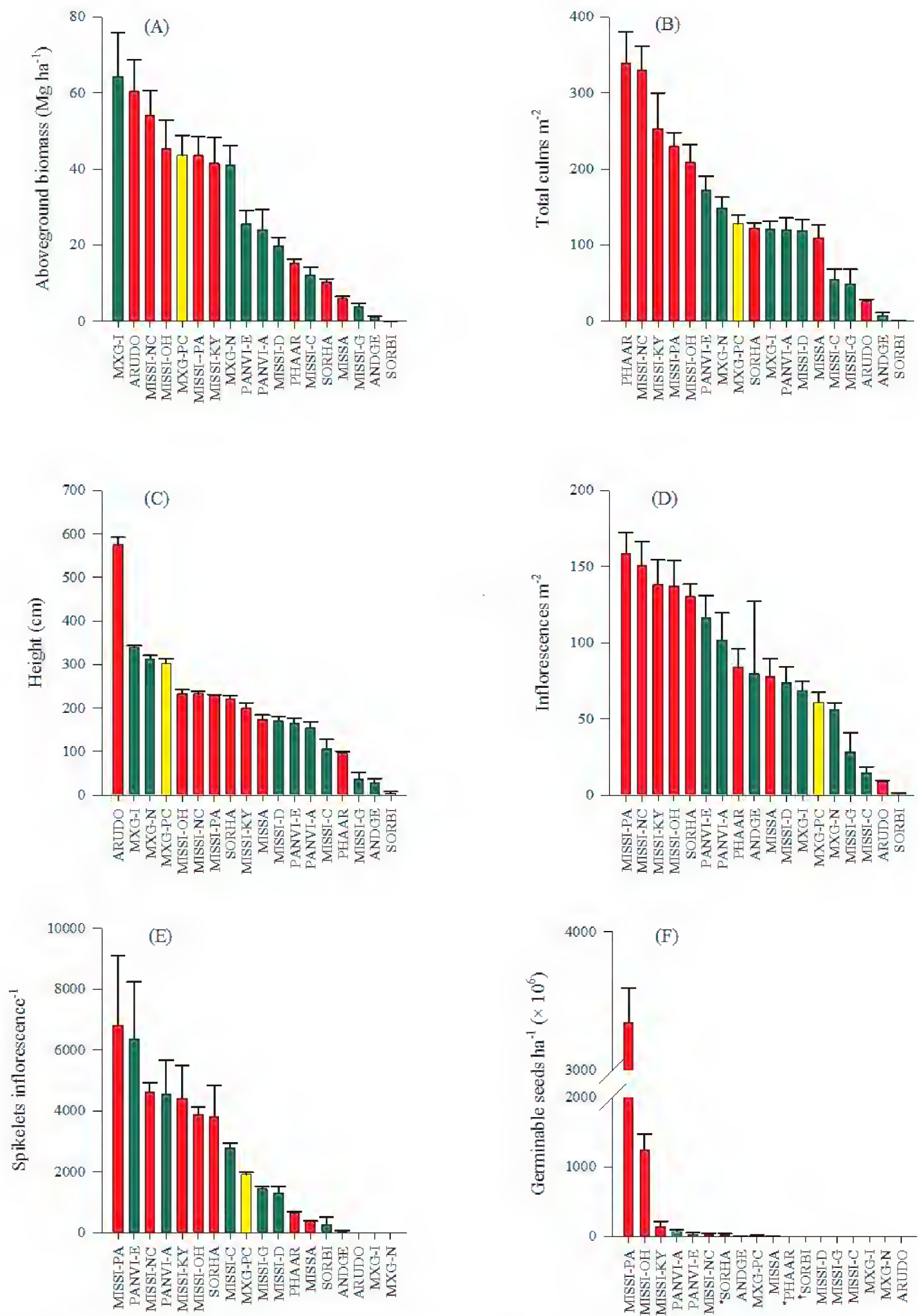
**Figure 2.** Mean culms (A), inflorescences (B), aboveground biomass (C), and height (D) for 10 species (20 total accessions) observed over three growing seasons in Blacksburg, VA.

### Year 3

Local spread of positive control species *S. halepense*, *P. arundinacea*, and *M. sacchariflorus* increased in year three in both Lc and Hc receiving areas. For the first time we observed seedlings of *P. virgatum*, *A. gerardii* and ornamental cultivars of *M. sinensis* (negative controls), ‘PowerCane’, and naturalized accessions of *M. sinensis* (positive controls) outside the cultivated plot in both Lc and Hc receiving areas.

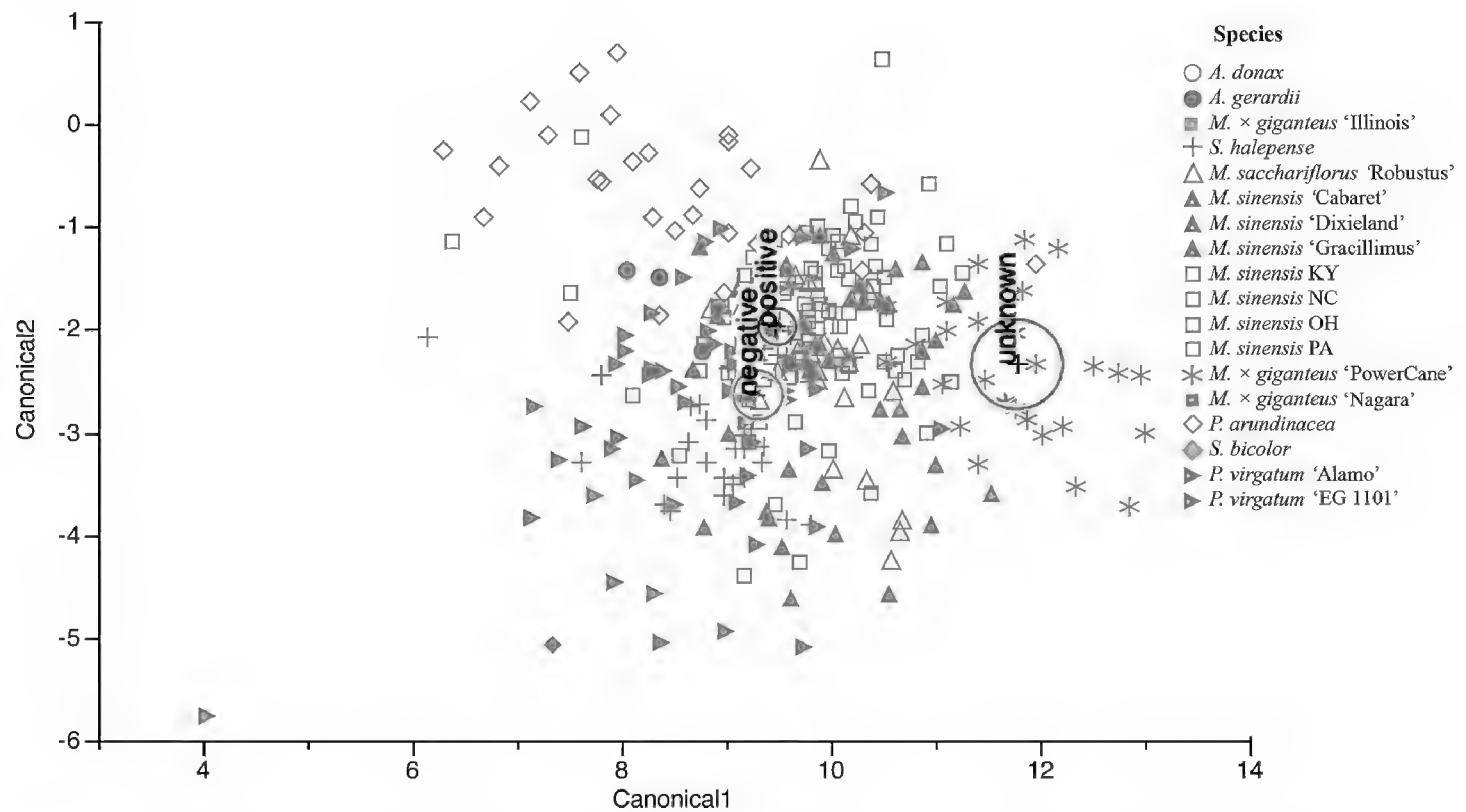
We saw no influence of either competition or disturbance on biomass, culm number, height, or inflorescence number in the third growing season (Table 2). ‘PowerCane’ was taller ( $325 \pm 12$  cm) and produced greater aboveground biomass ( $43.7 \pm 6.8$   $\text{Mg ha}^{-1}$ ) than both positive and negative control groups (Figure 2). However, our





**Figure 3.** The ranked aboveground biomass (A), culms (B), height (C), inflorescences (D), spikelets (E), and germinable seeds (F) for 18 accessions (two year-old *Saccharum*, spp. were omitted), recorded at the end of the third growing season. Accessions marked with \* indicate that seeds appeared immature at time of germination testing, and † indicate that seeds had extreme fungal and insect damage at the time of harvest.



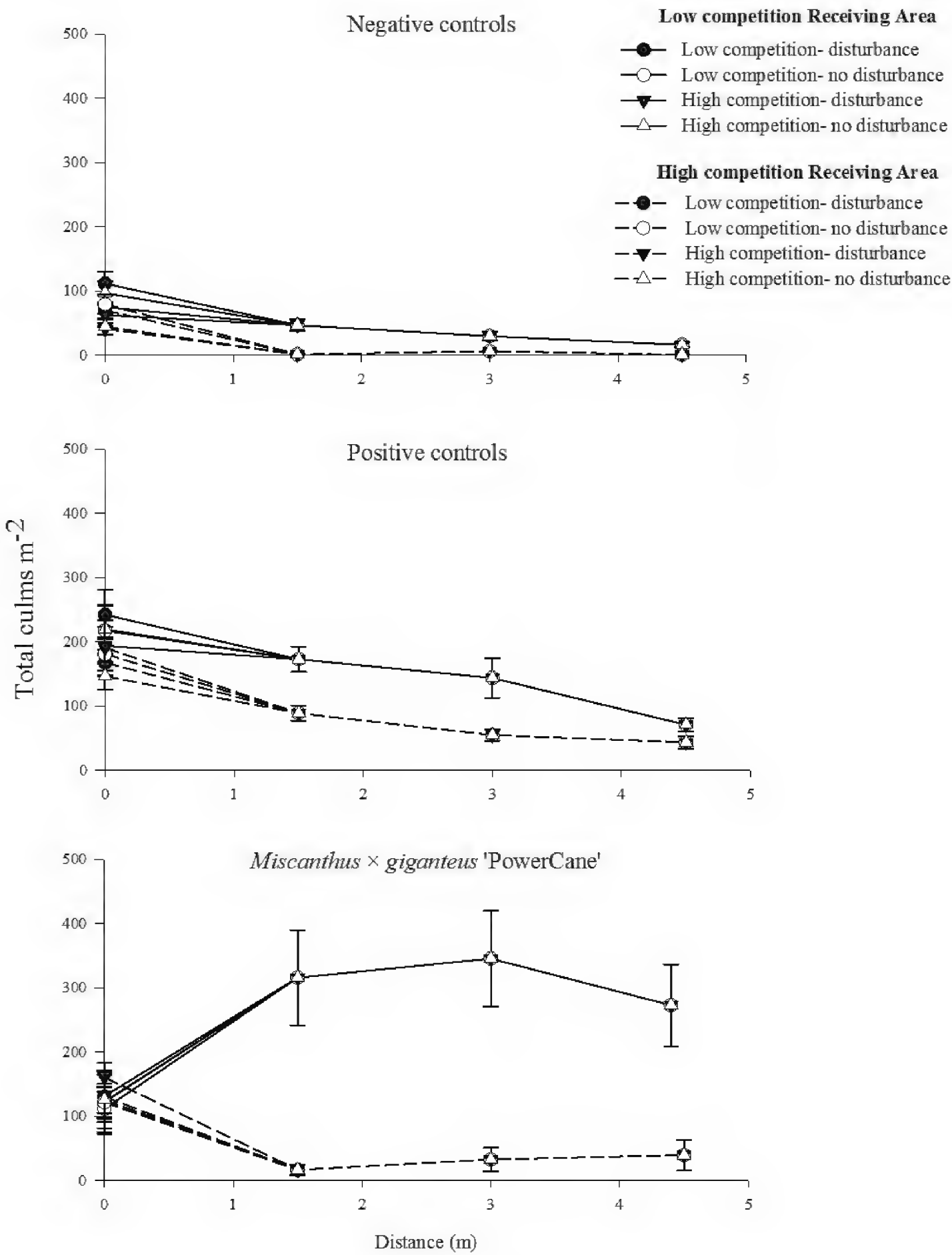


**Figure 4.** Canonical discriminant analysis plot for the species invasiveness groupings positive controls, negative controls and *M. x giganteus* 'PowerCane'. The fitness parameters biomass, culm and inflorescence number, height and spikelet production were used as predictors.

positive controls produced 52% more culms  $\text{m}^{-2}$  and 74% more inflorescences  $\text{m}^{-2}$  than 'PowerCane' (Figure 2). 'PowerCane' produced  $2,163 \pm 80$  spikelets inflorescence $^{-1}$ , significantly fewer than our positive control group ( $3,226 \pm 534$  spikelets inflorescence $^{-1}$ ) (Figure 3). 'PowerCane' seeds also exhibited low germinability with only one of 300 spikelets germinating after four weeks. Alternatively, two naturalized accessions of *M. sinensis*, PA and OH, had the highest seed germination rates (31% and 16% respectively) (Figure 3). The rank of performance varied among accessions and invasive groups among the functional traits recorded (Figure 3). When our functional traits were used as predictors, canonical discriminant analysis failed to show clustering of our invasive groups, with a predictive value < 62% (Figure 4).

In all cases, seedling or vegetative spread into adjacent receiving areas was greater in the Lc receiving area compared with the Hc receiving area (Figure 5). A significant interaction between invasive groups and receiving area competition was observed at the 0 to 1.5 m ( $P < 0.001$ ) and 1.5 to 3 m ( $P = 0.04041$ ) distances from the cultivated plot (Figure 5). While the greatest number of culms  $\text{m}^{-2}$  were observed in the 'PowerCane' Lc receiving areas, numbers were not different from those found in the positive control Lc plots. In the Hc receiving areas, culms  $\text{m}^{-2}$  produced by 'PowerCane' was equivalent to that of the negative control group. It should also be noted that, at no time were any of the sterile clones in this study (*A. donax* or *M. x giganteus* 'Illinois' and 'Nagara') observed to spread vegetatively outside of their associated planted plot.





**Figure 5.** Total number of vegetative and seedling culms m<sup>-2</sup> observed within the cultivated plot (distance 0 m), and in the Lc, low competition (intensive weed management) and Hc, high competition (no weed management) receiving areas from a distance of 0.1 to 4.5 m from the cultivated plot, after three growing seasons.



## Discussion

All taxa in our study established under all treatment conditions, and all fertile crops produced offspring, with the exception of the two *Saccharum* spp., which were only grown for two years. Despite enhanced traits for cold tolerance, these cultivars may have been well beyond their suitable geographic range in Blacksburg, VA (Barney and DiTomaso 2011) because they were the only species in this study to decrease in biomass (1300% Mg ha<sup>-1</sup>) and culm number (380%), as well as fail to produce inflorescences; however, the lack of inflorescences production was not unexpected as several abiotic factors are responsible for inhibiting and inducing tasseling, such as temperature and photoperiodism (LaBorde 2007).

Though all of the perennial species in our study have the ability to spread vegetatively, local spread was equivocal. Despite the three year clonal expansion of sterile *M. × giganteus* ‘Illinois’ (0.23 m<sup>2</sup> plant<sup>-1</sup> increase in area) and *A. donax* (0.28 m<sup>2</sup> plant<sup>-1</sup> increase in area), this did not contribute to nascent plants outside the cultivated plot. Unlike the culms of most species in our study, which die back at the end of each growing season, the culms of *A. donax* remain dormant during the winter months (Saltonstall et al. 2010). *Arundo donax* produced 33 ± 1 nodes culm<sup>-1</sup>; the majority of which produced new axial shoots each growing season. Despite overwhelming evidence of *A. donax* clonal spread in warm riparian or coastal freshwaters of the southwestern United States (Bell 1997; Quinn and Holt 2008; Seawright et al. 2009); the numerous culms we observed, bending to the ground at the perimeter of our plots, failed to root and produce new ramets; so called layering (Boland 2006). It has been suggested that the probability of a plant becoming invasive increases with the ability to reproduce vegetatively (Kolar and Lodge 2001). The only species in our trial for which vegetative reproduction appeared to contribute to invasive spread were *S. halepense*, *M. sacchariflorus*, and *P. arundinacea* (all positive controls), all of which began to spread from the planted plots in the second growing season. Conversely, Pyšek and Richardson (2007) argue that while vegetative traits may benefit persistence, the ability to spread may be hindered by vegetative reproduction, especially if seed production is limited or absent. In agreement with our results, all of the caespitose grasses in our study failed to spread vegetatively beyond the borders of the planted plot under our growing conditions.

The production of fertile seed enhanced the ability of many species to spread, but only locally. *Sorghum halepense* was the only accession to have large numbers of first season inflorescences (Figure 2B), and so it is not surprising that this was the only species to generate seedling volunteers in the second year. In contrast, most taxa increased inflorescence production in the second season, extending the range of species detected as seedlings in the receiving areas in the third season. With the exception of ‘PowerCane’, inflorescence numbers continued to increase from the second to the third growing season, suggesting that propagule pressure in the receiving plots did not reach a plateau during the experiment. While other factors such as habitat invasibility and timing are essential to invasive success (Barney and Whitlow 2008), this dramatic increase in propagule pressure facilitated establishment of ‘PowerCane’ seedlings in



both the Lc and Hc receiving area, though recruitment varied dramatically between the habitats. Inflorescence production and therefore the total number of spikelets plot<sup>-1</sup> in our three invasive groups increased between year one and year three. Even small increases in propagule pressure can result in a substantial increase in invasion pressure even in inhospitable environments (Davis 2009), which likely contributed to the observed lag.

The more individuals released into an environment the higher the probability that some propagules will endure environmental barriers and overcome stochastic biotic and abiotic factors (Blackburn et al. 2009). The number of introduced individuals, therefore, has a substantial influence on establishment success (Lockwood et al. 2009). Seeds of *A. gerardii*, *P. virgatum* and *P. arundinacea* are known to have variable dormancy and potentially low seedling vigor (Beckman et al. 1993; Lewandowski et al. 2003; Parrish and Fike 2005; Smart et al. 2003), which agreed with our observations in the establishment year. *Miscanthus sacchariflorus* (positive control) has been reported to have low seed set, ~746 viable seeds mature plant<sup>-1</sup>, with population growth predominantly due to vegetative spread (Madeja et al. 2012). Despite these assumptions, 1% of tested *M. sacchariflorus* seeds did germinate, which was greater than seed germinability of 'PowerCane' (0.3%) and *M. sinensis* NC (0.6%). Previous research has shown ornamental cultivars of *M. sinensis* are extremely variable, ranging from 190,000 seeds plant<sup>-1</sup> to 3,100 in 'Gracillimus', 785 in 'Dixieland' and 0 filled spikelets plant<sup>-1</sup> in 'Cabaret' (negative controls) (Madeja et al. 2012; Meyer and Tchida 1999). Results from our seed germination testing support these conclusions in which no ornamental *M. sinensis* seeds germinated; however, we observed high variability (31% to 0.6%) in germinability for naturalized *M. sinensis* accessions (Figure 3). Dougherty et al. (2014) showed that ~44% of seed from weedy accessions of *M. sinensis* germinated in a laboratory setting. Previous research indicates that both inflorescence and spikelet production in almost all *M. sinensis* cultivars are positively correlated with plant hardiness zone (Madeja et al. 2012; Meyer and Tchida 1999; Wilson and Knox 2006); indicating that invasion pressure for these species may vary with latitude.

After three years of growth, all naturalized accessions of *M. sinensis* produced a greater number of inflorescences m<sup>-2</sup> and more spikelets inflorescence<sup>-1</sup> than 'PowerCane' (Figure 3). In this study, 'PowerCane' produced ~1.3 billion spikelets ha<sup>-1</sup> in the third growing season, which would yield 3.9 million viable seed ha<sup>-1</sup> given the 0.3% germination rate. It is possible that we saw greater numbers of seed produced than in a production field; like *P. virgatum* (Martinez-Reyna and Vogel 2002), *Miscanthus* spp. are self-incompatible (Hirayoshi et al. 1955). The genetic diversity among the accessions and species in our study, ideal for outcrossing species, may have led to inflated seed production (Madeja et al. 2012) compared to the relative genetic homogeneity common in commercial crops. In this case genetic variability not only increases the likelihood of seed production, but also has the potential to enhance establishment success and increase the habitat range of exotic species (Lockwood et al. 2005).

Surprisingly, none of the accessions were affected by the level of competition, which not only contradicts much of the literature suggesting the need for weed man-



agement at establishment (Lewandowski et al. 2003), but supports the conclusion that these species are fast growing and highly competitive (Raghu et al. 2006). The high seedling establishment of ‘PowerCane’ in the Lc and lower seedling establishment numbers in the Hc, appears to contradict our recent findings in which only 0.1% of emerged ‘PowerCane’ seedlings survived after six months (Smith and Barney 2014b). However, our earlier study indicated the majority of ‘PowerCane’ seedlings emerged in areas of available bare ground and low resident plant competition such as agricultural fields and forest understories. However, annual weed species dominated our Hc receiving area, which created open spaces and the availability of bare ground during the winter months and early spring, coinciding with annual seed dispersal for many of these late flowering species. The small, light seed and ciliate lemma (Gleason 1952) of *Miscanthus* spp. is valuable for dispersal. However, the ciliate lemmas appear to interfere with the ability of *Miscanthus* seed to make important soil contact required for germination, which is evident in areas of high resident plant competition and litter (Smith and Barney 2014b). According to Quinn et al. (2011), the anemochorous *M. sinensis* spikelets have been shown to disperse an average of 50 m. Therefore, open areas of low resident plant competition near production sites will likely be the most susceptible, suggesting that our Lc plots represent a worst case scenario. This information will be critical for identifying susceptible habitats near cultivated fields and will be important considerations for management.

Despite the utility of trait-based research for helping to make associations and guide management, traits do not confer absolute predictability. Invasions will always be contingent on a number of interacting factors (Barney et al. 2008; Dredovsky et al. 2012). Hence, our experimental design was critical in the interpretation of our results. Clearly this geographic location, habitat and treatment factors were ideal for a species such as *S. halepense*; a species intentionally introduced for agronomic purposes, which now flourishes in agricultural and anthropogenic systems of the southeastern U.S. (Warwick and Black 1983). However, despite the reputation of *A. donax* as an aggressive riparian invader, this species appeared to be constrained by the non-riparian landscape.

The selection of our invasive and noninvasive taxa was a novel methodology used to make important relative comparisons. The ten species selected in this study are of similar life form, and all of them, including *S. halepense* (Nackley et al. 2013), have the potential to be used as a feedstock in bioenergy production. Comparisons of ‘PowerCane’ to specific taxa, *S. halepense* for example, across an invasive spectrum, provided meaningful information. However, making all pairwise comparisons would not be ecologically and statistically meaningful; therefore, our goal was to also find and examine broader trends within invasive groups. Interestingly, our positive control species were selected on the basis of a past history of invasiveness, a robust predictor of future invasiveness (Davis 2009). However, multivariate analysis failed to indicate a relationship between our measured functional traits and our invasive groups (Figure 4). After three growing seasons, our ranking of fitness metrics also fails to show the anticipated consistent gradient of positive (high fitness) to negative (low fitness). This suggests that functional traits vary in their relationship to invasiveness (Dredovsky et al. 2012).



However, meta-analysis reveals that traits such as fitness, size, and growth rate were significantly higher for exotic invasive species when compared with the traits values of non-invasive species (van Kleunen et al. 2010). It is imperative to remember that invasiveness is not a “one size fits all” scenario, which is made evident in our study. Traits vary with life stage and environmental conditions and the importance of any given trait will therefore also vary (Davis 2009; Pyšek and Richardson 2007). While our positive and negative invasive groups may not have clustered as we hypothesized; the use of numerous species and accessions allows us to evaluate the usefulness of traits across an invasive spectrum.

Unfortunately the loss of our Kentucky and Alabama sites limits our ability to generalize across a broader geographic range. The ability to observe relative comparisons across a gradient of species reinforces the fact that it is the important interaction of species and habitat that result in invasive populations (Barney and Whitlow 2008). Our results indicate that several candidate feedstocks have the ability to move from the cultivated field, but it should be noted that we only recorded spread to < 5 m from the field edge in three years. On average, more than 100 culms m<sup>-2</sup> were observed in every measured quadrat in the Lc receiving area of *S. halepense*, naturalized *M. sinensis*, *P. virgatum* and ‘PowerCane’. Conversely, only *S. halepense* and naturalized *M. sinensis* maintained high numbers in the Hc receiving area when *P. virgatum* and *M. × giganteus* ‘PowerCane’ showed a dramatic decrease in culm numbers.

## Conclusion

Bioenergy crop movement beyond the cultivated field would not be novel to agronomic crops because feral escapes are known for most row crops (DiTomaso and Healey 2007). Yet due to their economic and social importance, crops are not frequently discussed in the invasion literature. Our experiment covers the first three years of the establishment phase, and unexpected and nonlinear changes may manifest in subsequent stages. Our results suggest that at least short-range movement away from the cultivated field is probable for fertile bioenergy feedstocks. It should be noted that no species in this study were detected outside our trial boundary, which predominately consisted of a mowed perennial border. Further study, across broader geographic locations and continued research will be necessary to further determine acceptable risk and management planning.

The use of ‘PowerCane’ or other fertile *M. × giganteus* germplasm could improve grower adoption but the invasive potential and ecosystem impacts of widespread cultivation still require further evaluation including the determination of climatic limitations of *M. × giganteus* and other bioenergy crop seedlings. The ability to contextualize our results suggests that *M. × giganteus* ‘PowerCane’ did not have the highly competitive seedling establishment potential of *S. halepense*. Alternatively, in this growing region sterile cultivars provide a lower risk option, but require additional economic investment. The scrutiny that has been applied to bioenergy crops indicates that we

have moved beyond the once cavalier approach toward species introduction. These efforts should continue in order to reduce unwanted and unintentional invasive spread. Nascent populations or seedlings may be easily overlooked. However, management at the seedling or early growth stage will likely increase the chances of successful control (Stephens and Sutherland 1999). Our relative methodology and results from this study can help us prepare for industry development while helping to minimize risk and mitigate invasive spread.

## Acknowledgments

Thanks to Ryan Dougherty, Eugene Dollete, John Halcomb, Matt Ho, Daniel Tekiela, Elise Benhase, Phillip Cox, and Carissa Ervine for help during installation, data collection, and harvest of this experiment. Thanks to Mendel Biotechnology, Inc for donation of *M. × giganteus* ‘PowerCane’ and ‘Nagara’ plugs and Dr. Erik Sacks for naturalized *M. sinensis* seed. The USDA graciously provided the two *Saccharum* spp.

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